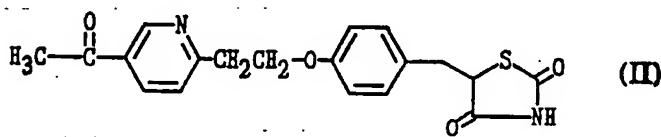
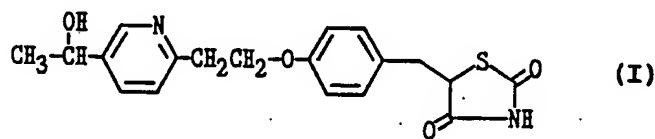




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US93/03569</p> <p>(22) International Filing Date: 21 April 1993 (21.04.93)</p> <p>(30) Priority data: 07/878,952 5 May 1992 (05.05.92) US 07/882,056 13 May 1992 (13.05.92) US</p> <p>(60) Parent Application or Grant (63) Related by Continuation US Filed on 07/882,056 (CO/N) 13 May 1992 (13.05.92)</p> <p>(71) Applicant (for all designated States except US): THE UP-JOHN COMPANY [US/US]; 301 Henrietta Street, Kalamazoo, MI 49001 (US).</p>		<p>(72) Inventors; and (75) Inventors/Applicants (for US only) : JOHNSON, Roy, A. [US/US]; 2122 Frederick Avenue, Kalamazoo, MI 49008 (US). MARSHALL, Vincent, P. [US/US]; 203 Paisley Court, Kalamazoo, MI 49007 (US). LI, Grace, P. [US/US]; 5128 Grosse Pointe, Kalamazoo, MI 49001 (US).</p> <p>(74) Agent: GAMMILL, Martha, A.; Corporate Intellectual Property Law, The Upjohn Company, 301 Henrietta Street, Kalamazoo, MI 49001 (US).</p> <p>(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p>	

(54) Title: A PROCESS FOR PRODUCING PIOGLITAZONE METABOLITES



(57) Abstract

The present invention provides a method for making pharmaceutical compounds. More particularly the present invention provides a new method for making oxygenated pioglitazone derivatives of formulas (I) and (II), which are metabolites of pioglitazone and useful for the treatment of diabetes and as insulin sensitizing agents.

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A PROCESS FOR PRODUCING PIOGLITAZONE METABOLITES  
FIELD OF INVENTION

The present invention provides a new method of making organic compounds. In particular, the present invention provides a new method for making oxygenated pioglitazone derivatives. These compounds are metabolites of pioglitazone and are useful for the treatment of diabetes and as insulin sensitizing agents.

BACKGROUND

Pioglitazone hydrochloride ((+)-5-[4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione monohydrochloride), a thiazolidinedione derivative, is currently under clinical evaluation and is expected to effectively ameliorate the abnormal glucose and lipid metabolism associated with noninsulin dependent diabetes mellitus (NIDDM) or obesity (cf. Y. Momose et al., *Chem. Pharm. Bull.*, 39:1440 (1991)).

International application, PCT/US92/02566, filed 6 April 1992 (WO 92/18501, published 29 October 1992), discloses thiazolidine derivatives, including 5-[4-[2-(5-acetyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione and 5-[4-[2-[5-(1-hydroxyethyl)-2-pyridyl]ethoxy]benzyl]-2,4-thiazolidinedione, which are pioglitazone metabolites.

Another thiazolidine derivative undergoing clinical studies as a hypoglycemic agent is englitazone sodium, 5-([3,4-dihydro-2-(phenylmethyl)-2H-1-benzopyran-6-yl]methyl)-2,4-thiazolidinedione sodium salt) (cf. D.A. Clark et al., *J. Med. Chem.*, 34:319-325 (1991)).

Ciglitazone ((+-)-5-[4-[(1-methylcyclohexyl)methoxy]benzyl]-2,4-thiazolidinedione) is characteristic of a class of thiazolidine antidiabetic agents which lower blood glucose in animal models of NIDDM, while actually reducing circulating concentrations of insulin. This is believed to be accomplished by improving the responsiveness of the peripheral tissues to insulin. See, e.g., Chang, et al., *Diabetes* 32:830-838 (September 1983).

CS-045 is an antidiabetic, thiazolidinedione derivative. Its activity and preparation are described in *Drugs Fut.* 1991, 16(9).

Also, thiazolidine derivatives useful for the treatment of diabetes are described in U.S. patents 4,287,200; 4,687,777; and 4,572,912. Their effect on insulin resistance are described, e.g., in Chang et al., *Diabetes* 32:839-845 (1983) and Chang et al., *Diabetes* 32:830-838 (1983).

The chemical synthesis of several of these thiazolidinedione derivatives have proved problematic and inefficient. In particular, the chemical synthesis of the pioglitazone metabolites includes long, tedious synthetic routes with poor or highly variable yields. What is needed in the art is an easier, more efficient method for performing the synthesis of these compounds.

INFORMATION DISCLOSURE

International application, PCT/US92/02566, filed 6 April 1992 (WO 92/18501, published 29 October 1992), discloses thiazolidine derivatives, including 5-[4-[2-(5-acetyl-2-

pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione and 5-[4-[2-[5-(1-hydroxyethyl)-2-pyridyl]ethoxy]benzyl]-2,4-thiazolidinedione, which are pioglitazone metabolites, and describes their chemical synthesis.

U.S. Patent 4,687,777 and EP 193-256, published 9 March 1986, disclose new 5-((ethyl-  
5 pyridyl) ethoxy) benzyl thiazolidine-dione compounds, including pioglitazone and  
pharmacologically acceptable salts, which have hypoglycaemic and hypolipaemic activity and  
thus are useful for treating diabetes, and describes the chemical synthesis of these compounds.  
European published application 0 508 740 discloses N-oxide thiazolidinedione  
derivatives, including the compound of formula IV, as shown in the Formula Chart below,  
10 having hypoglycemic and hypolipidemic activity.

U.S. Patent 4,444,779 and EP 0 008 203 disclose new thiazolidine-dione compounds,  
including ciglitazone and pharmacologically acceptable salts thereof, which have hypoglycaemic  
and hypolipaemic activity, and describes the chemical synthesis of these compounds.

Derwent Abstract 88-057989/09 of EP 257 781 discloses new substituted pyridine  
15 compounds used in the preparation of hypoglycaemic and hypolipidaemic pyridyl-ethoxy-  
methylphenyl thiazolidine-dione compounds.

The use of various microorganisms for the oxygenation of certain organic molecules is  
known in the art, for example, in the following references: R.A. Johnson, in "Oxidation in  
Organic Chemistry, Part C," ed. W.S. Trahanovsky, Academic Press, N.Y., 1978, pp. 131-210;  
20 H.G. Davies, et al., "Biotransformations in Preparative Organic Chemistry", Academic Press,  
N.Y., 1989, pp. 157-219.

U.S. Patent 2,602,769 discloses the oxygenation of steroids by mucorales fungi.  
In the process of the present invention, a microorganism may be used to deoxygenate  
and then oxygenate a compound.

25 Y. Momose et al., Chem. Pharm. Bull., 39:1440 (1991); K. Meguro et al., Japan. Patent  
139182 (1988); Chem. Abstr., 109:6504h (1988); and T. Sohda et al., Arzneim.-Forsch./Drug  
Res. 40 (I), Nr. 1:37 (1990); disclose the chemical synthesis and biological activity of various  
thiazolidinedione derivatives, including pioglitazone.

D.A. Clark et al., J. Med. Chem., 34:319-325 (1991) discloses the chemical synthesis of  
30 substituted dihydrobenzopyran and dihydrobenzofuran thiazolidine-2,4-diones, including  
englitazone.

Drugs Fut. 1991, 16(9) discloses the multistep process, via carbon alkylation, for the  
preparation of the thiazolidinedione CS-045.

PCT/US92/10329, filed 4 December 1992, discloses a new reduction method for making  
35 thiazolidinedione derivatives, particularly ciglitazone, pioglitazone and englitazone.

#### SUMMARY OF THE INVENTION

The present invention particularly provides:

A process for producing a compound of the formula I or II which comprises:

the biotransformation of a compound of the formula IV using a microorganism in an aqueous nutrient medium containing an assimilable source of carbon and an assimilable source 5 of nitrogen under aerobic conditions;

A biologically pure culture of the microorganism *Streptomyces hygroscopicus*, characterized as the strain NRRL 18975;

A method of using a compound of the formula IV to produce a compound of the formula I or II which comprises:

10 adding the compound of formula IV to a metabolizing culture of a microorganism;

A method of using a microorganism *Streptomyces hygroscopicus*, strain NRRL 18975, or a mutant thereof, to produce a compound of the formula I or II which comprises:

adding a compound of the formula IV to a metabolizing culture of the microorganism; and

15 A method of deoxygenating the N-oxide of a pyridine ring and oxygenating the 5-position ethyl side chain of the pyridine ring in a compound of the formula IV to produce a compound of the formula I or II which comprises:

adding the compound of formula IV to a metabolizing culture of a microorganism.

By "biotransformation" is meant the use of microorganisms and/or isolated, partially 20 purified enzymes for the conversion of a given substrate into a useful product. H.G. Davies, et al., "Biotransformations in Preparative Organic Chemistry", Academic Press, N.Y., 1989, p. IX.

By "metabolizing" is meant carrying out the processes of metabolism. A definition of metabolism may be found in A.L. Lehninger, "Principles of Biochemistry", Worth Publ., New York, 1982, p. 333.

25 The pioglitazone metabolites produced by the process of the present invention are 5-[4-[2-(5-acetyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione and 5-[4-[2-[5-(1-hydroxyethyl)-2-pyridyl]ethoxy]benzyl]-2,4-thiazolidinedione. They are represented as Formulas I and II in the Formula Chart below. These compounds have excellent hypoglycemic and hypolipidemic activity and thus are useful as therapeutic agents to treat diabetes, hyperlipidemia and the like.

30 These compounds, their pharmacologically acceptable salts or pure stereoisomeric forms can be used for the treatment of diabetes of mammals, including man, as they are or by combining them with known pharmacologically acceptable carriers, excipients, fillers and the like. Normally, such a compound, its pharmacologically acceptable salt or stereoisomeric form can be administered orally in the form of, for example, tablets, capsules including soft capsules 35 and micro capsules, powders, granules or the like. If necessary, it can also be administered parenterally in the form of injectable solutions, suppositories, pellets or the like. In the case of

oral administration, preferably, it is administered one to three times a day in a daily dose of 0.05 to 100 mg/kg, and preferably 0.05 to 10 mg/kg. These pioglitazone metabolites, their biological activity and chemical synthesis is further described in International application,

PCT/US92/02566, filed 6 April 1992 (WO 92/18501, published 29 October 1992), which is

5 hereby incorporated by reference herein.

Previously, the chemical synthesis of these pioglitazone metabolites proved problematic and inefficient. In particular, their synthesis includes long, tedious routes with poor or highly variable yields. Surprisingly and unexpectedly, the present invention provides a new, more efficient method for producing these compounds. By using the new method of the present 10 invention, the metabolites are produced by adding pioglitazone of Formula III, pioglitazone hydrochloride of Formula V and/or pioglitazone N-oxide of Formula IV, as shown in the Formula Chart below, to growing cultures of microorganisms. The species *Streptomyces hygroscopicus* is preferred for effecting this transformation and the novel strain NRRL 18975 is particularly preferred. Pioglitazone hydrochloride and pioglitazone N-oxide are preferred as 15 starting materials, with pioglitazone N-oxide being most preferred as the starting material because of its better solubility characteristics in comparison with pioglitazone. This new method is faster and easier and results in improved yield of the desired metabolites.

For example, Upjohn soil isolate 02179 (UC® 11099) deoxygenates the pyridine-N-oxide of pioglitazone-N-oxide and oxygenates its 5-position ethyl side chain. It also oxygenates 20 the 5-position ethyl side chain of pioglitazone. The culture grows well and produces a white aerial mass which becomes hygroscopic and turns dark gray to black on some media. The mature spore chains are long and form tight spirals. The spore surface is rugose. It has wide range of growth temperature and utilizes most carbon sources. The whole-cell hydrolysate contains LL-diaminopimelic acid. Based on the morphological and cultural characteristics and 25 the whole-cell hydrolysate analysis of the culture, the species classification *Streptomyces hygroscopicus* has been determined for the culture. The characterization of this microorganism is further described in Example 2 below. A subculture of this microorganism was deposited under the provisions of the Budapest Treaty on 6 May 1992 in the permanent collection of the Northern Region Research Center, ARS; U.S. Dept. of Agriculture; Peoria, Illinois, USA. Its 30 accession number is NRRL 18975.

Table 4 lists other strains of *Streptomyces hygroscopicus* useful in the process of the present invention. The Upjohn Culture Collection (UC®) Number for these microorganisms is given. Since these microorganisms have been deposited in a permanent collection of a recognized depository, their official accession number or deposit number is also given. The 35 following two microorganisms are also useful in the process of the present invention: *Rhizopus Japonicus* (UC No. 4842; Deposit No. IFO 4758) and *Streptoverticillium sp.* (UC No. 2064;

Deposit No. NRRL 21066). The use of these microorganisms in the process of the present invention results in the production of one or both of the compounds of the Formulas I and II.

Pioglitazone metabolites (Formulas I and II) are produced when *Streptomyces hygroscopicus* is fermented in an aqueous nutrient medium under submerged aerobic conditions  
5 in the presence of pioglitazone, pioglitazone hydrochloride and/or pioglitazone N-oxide. Typically the microorganism is fermented in a nutrient medium containing a carbon source and a proteinaceous material. Preferred carbon sources include glucose, brown sugar, sucrose, glycerol, starch, cornstarch, lactose, dextrin, molasses, and the like. Preferred nitrogen sources include cottonseed flour, corn steep liquor, yeast, autolyzed brewer's yeast with milk solids,  
10 soybean meal, cottonseed meal, cornmeal, milk solids, pancreatic digest of casein, distillers' solids, animal peptone liquors, meat and bone scraps, and the like. Combinations of these carbon and nitrogen sources can be used advantageously. Trace metals, for example, zinc, magnesium, manganese, cobalt, iron and the like need not be added to the fermentation medium since tap water and unpurified ingredients are used as medium components.  
15 Production of pioglitazone metabolites can be induced at any temperature conducive to satisfactory growth of the microorganism between about 23° and 32° C and preferably at about 28°C. Ordinarily, optimum production of the pioglitazone metabolites is obtained in about 1 to 4 days after addition of the pioglitazone or pioglitazone-N-oxide to the growing culture, and preferably in about 1.5 days. The fermentation broth normally remains weakly basic (pH 7.4 -  
20 9.0) during the fermentation. The final pH is dependent, in part, on the buffers present, if any, and in part, on the initial pH of the culture medium. It is advantageously adjusted to about pH 6.5-7.5, and preferably 7.2, prior to sterilization.

When growth is carried out in shake flasks or large vessels and tanks, it is preferable to use the vegetative form, rather than the spore form, of the microorganism for inoculation to  
25 avoid a pronounced lag in the production of the pioglitazone metabolites and the attendant inefficient utilization of the equipment. Accordingly, it is desirable to produce a vegetative inoculum in an aqueous nutrient medium by inoculating this medium with an aliquot from a soil or a slant culture. When a young, active vegetative inoculum has thus been secured, it is transferred aseptically to other shake flasks or tanks. The medium in which the vegetative inoculum  
30 is produced can be the same as, or different from, that utilized for the production of the antibiotics, as long as it is such that adequate growth of the microorganism is obtained.

A variety of procedures can be employed to isolate and purify the pioglitazone metabolites from the fermentation broth, for example, by chromatographic adsorption procedures followed by elution with a suitable solvent, column chromatography, partition chromatography,  
35 and crystallization from solvents and combinations thereof.

In the preferred recovery process, the pioglitazone metabolites are extracted from the

whole beer. Column chromatography techniques, preferably over silica gel, are used to perform the initial purification. Final purification of the pioglitazone metabolites is achieved by chromatography and crystallization from organic solvents.

It will be apparent to those skilled in the art that compounds of Formulas I and II  
5 contain several asymmetric carbons. All of the enantiomeric and stereoisomeric forms of the compounds of Formulas I and II are included within the scope of the present invention.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is seen more fully by the examples below, but these examples are not to be construed as limiting the scope of this invention.

10 PREPARATION 1 Preparation of Pioglitazone-N-oxide (also known as (+)-5-[4-[2-(5-ethyl-  
2-pyridyl-N-oxide)ethoxy]benzyl]-2,4-thiazolidinedione) Formula IV  
(Formula Chart)

A mixture of pioglitazone (48.33 g), chloroform (1.2 l), and methanol (375 ml) is stirred at room temperature. m-Chloroperbenzoic acid (41.90 g of 60% technical grade, 25.1 g) is added as the solid in three portions over a period of 45 minutes. The stirred mixture becomes a clear solution within one hour of the addition of m-chloroperbenzoic acid. The solution is stirred overnight at room temperature and then worked up extracting and washing sequentially with saturated sodium bicarbonate (600 ml) plus water (500 ml), with aqueous sodium sulfite (10 g in 200 ml of water), and with water (500 ml). The hazy solution is dried (sodium sulfate), filtered, and concentrated under reduced pressure. The oily, warm residue is immediately dissolved in acetonitrile (350 ml) and crystallization usually begins immediately. Crystallization is allowed to proceed for 24 hours after which colorless crystals of IV (42.11 g) are collected.

Recrystallization from ethyl acetate gives IV as colorless crystals.  
25 Physical characteristics are as follows:  
M.P. 159-161°C;  
<sup>1</sup>H NMR (CD<sub>3</sub>OD) δ 8.23, 7.49, 7.44, 7.15, 6.86, 4.67, 4.38, 3.35, 3.30, 3.07, 2.67,  
1.25.

Anal. Found: C, 60.82; H, 5.69; N, 7.40; S, 8.45.

30 PREPARATION 2 Protocol for Biotransformation of Pioglitazone (also known as (+)-5-[4-[2-(5-ethyl-  
2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione) Formula III,  
pioglitazone hydrochloride (also known as (+)-5-[4-[2-(5-ethyl-  
2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione monohydrochloride)  
Formula V and/or Pioglitazone N-oxide (also known as (+)-5-[4-[2-(5-  
35 ethyl-2-pyridyl-N-oxide)ethoxy]benzyl]-2,4-thiazolidinedione) Formula  
IV (Formula Chart)

A. Seed Fermentation

*Streptomyces hygroscopicus* (NRRL 18975)

Frozen agar plugs of NRRL 18975 (stored over liquid nitrogen) are aseptically transferred into a 100 ml vol of GS-7 (sterile) medium contained in a 500 ml large mouth shake flask. GS-7 is composed of Cereose and Pharmamedia, each added at 25 g/l of tap water. The pH of the medium is adjusted to pH 7.2 using ammonium hydroxide. The medium is sterilized by autoclaving for 30 min. Inoculated GS-7 is shaken at 250 rpm at 28° C for 72 hrs. This fermentation is used to inoculate the biotransformation process at a 5% rate.

B. Biotransformation Process

10 100 ml vols of GS-7 contained in 500 ml large mouth fermentation flasks are inoculated with the 72 hr seed fermentation at a 5% rate. The inoculated GS-7 is shaken at 250 rpm at 28° C for 48 hrs. At this time pioglitazone, pioglitazone hydrochloride or pioglitazone N-oxide dissolved in dimethylformamide (DMF) is added to the fermentation in a range between 20 and 60 mg/flask using no more than 0.3 ml DMF per flask. The fermentations containing  
15 Pioglitazone or Pioglitazone N-oxide are continued as previously indicated for 24-96 hrs.

EXAMPLE 1 Isolation of Metabolites, 5-[4-[2-[5-(1-hydroxyethyl)-2-pyridyl]ethoxy]benzyl]-2,4-thiazolidinedione and 5-[4-[2-(5-acetyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione, Formulas I and II (Formula Chart)

The contents of 120 flasks, each containing 100 ml fermentation as described in the  
20 protocol for biotransformation and which has been shaken at 28° C for 24 hrs following addition of pioglitazone-N-oxide (0.050 g per flask, total 6.0 g) in dimethylformamide (0.2 ml per flask, total 24 ml), are pooled in a 20 l carboy. The mixture is stirred vigorously with methylene chloride (methylene chloride, 7 l) for 3 hrs. The solvent layers separate slowly and after 24 hrs, the methylene chloride layer (1.75 l) is siphoned off under a slight vacuum. Additional  
25 methylene chloride (2 l) is added, the mixture is again stirred vigorously and this procedure continues through three more collections of methylene chloride extract (of 15 l of methylene chloride added to the mixture, about 10 l are collected). The methylene chloride extracts are dried (sodium sulfate), filtered, and concentrated. The oily residues are placed under a stream of nitrogen for 24-48 hrs, which removes much of the dimethylformamide that is carried into the  
30 extract. Upon standing for several days, solids precipitate in the oily residues. These are collected by using small amounts of methylene chloride to wash and transfer during the filtration process. In this way, a yellowish-brown solid (2.27 g) is collected. Examination of this solid by thin layer chromatography (TLC, silica gel plates, 10:1 methylene chloride-methanol; 1:1 acetone-hexane as solvents for TLC) shows the presence of materials with the same R<sub>f</sub> as  
35 authentic samples of compounds of formulas I and II as well as other components. The components of the solid extract are separated by a series of column chromatographies using a

silica gel-solid extract ratio of 50-125:1. Columns are eluted with either 4-5% methanol in methylene chloride or 30-60% acetone in hexane. From these chromatographic separations, there is isolated 5-[4-[2-(5-acetyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione (0.203 g), the compound of formula II, which is recrystallized from acetone-hexane to give colorless needles  
5 (0.147 g).

Physical characteristics are as follows:

Mp 113-115°C;  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ ) is identical to that of an authentic sample.

Also isolated is 5-[4-[2-[5-(1-hydroxyethyl)-2-pyridyl]ethoxy]benzyl]-2,4-thiazolidinedione (0.756 g), the compound of formula I, which is recrystallized from acetone-  
10 hexane to give colorless crystals (0.680 g).

Physical characteristics are as follows:

Mp 154-155°C;  $[\alpha]_D^{25.5} -12.4^\circ (\text{C}, 1.18 \text{ in ethanol})$ ;  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ ) is identical with the spectrum of an authentic sample.

The following similar experimental procedure resulted in improved yields of the  
15 compounds of formulas I and II:

A solution of pioglitazone-N-oxide (6.0 g.) in dimethylformamide (total volume of solvent and substrate, 24 mL) is divided equally among 120 flasks each containing 100 mL of medium and growing culture and shaking at 28°C is continued for 24 hrs. The contents of the flasks are pooled in a 20 L glass carboy, methylene chloride (6 L) is added, and the two-phase system is stirred vigorously with an air driven motor for 2 hrs. The solvent layers separate slowly and after about 24 hrs, the methylene chloride layer is siphoned off under a slight vacuum. The extraction process is repeated by adding fresh methylene chloride in the amount removed to the pooled fermentation, stirring vigorously, and removing methylene chloride by siphon. This extraction process is done a total of ten times. Extractions 1-7 (total volume 18.2  
25 L) are combined and extractions 8-10 (total volume 12.2 L) are combined.

Combined extracts 1-7 are dried (sodium sulfate), filtered, and concentrated. The residual material is chromatographed over silica gel (40-63  $\mu\text{m}$ , 400 g, preconditioned with *i*-propyl alcohol/ethyl acetate/hexane/glacial acetic acid (5/65/29.5/0.5)) packed in a 4.7 x 45 cm Michel-Miller column. The residue is applied to the column by first dissolving 1:1 chloroform-methanol, mixing with gravity grade silica gel (63-200  $\mu\text{m}$ , 35 g), and removing the solvent on the rotary evaporator under reduced pressure. The dry solid is placed in a second 4.7 x 45 cm Michel-Miller column held in a vertical position. The empty space in the column is filled with 40-63  $\mu\text{m}$  silica gel and the column is eluted with the same solvent mixture used to precondition the column. Fractions of 50 mL volume are collected and are pooled based on the results of tlc analysis.  
35

Fractions 21-33 are pooled and contain ketone of formula II and a small amount of

pioglitazone of formula III (total, 1.20 g). This material is chromatographed over 200 g of 40-63 $\mu$ m silica gel using ethyl acetate to apply the material to the column and *i*-propyl alcohol/ethyl acetate/hexane/ glacial acetic acid (5/65/29.5/0.5) to elute the column. Fractions of 30 mL volume are collected with fractions 11-15 containing the desired ketone of formula II 5 (0.955 g) still mixed with pioglitazone of formula III. The mixture is again chromatographed over 200 g of 40-63 $\mu$ m silica gel, eluting with chloroform/*i*-propyl alcohol/acetic acid (94/5/1) and collecting 40 mL fractions. Fractions 12-15 contain crystalline ketone of formula II (0.602 g) and fractions 19-26 contain impure pioglitazone of formula III (0.184 g). The combined fractions 12-15 are recrystallized from acetone-hexane, giving the ketone of formula II (0.511 10 g).

Physical characteristics are as follows:

Mp 113-115°C.

The  $^1\text{H}$  NMR spectrum in  $\text{CDCl}_3$  of the sample of ketone of formula II is identical to the spectrum of an authentic sample of ketone of formula II.

15 Fractions 34-40 are pooled and contain a mixture of ketone of formula II and alcohol of formula I (total, 0.41 g). This mixture is plated onto 8 g of silica gel and placed in line in front of a column of 200 g of 40-63  $\mu$ m silica gel. The system is eluted with chloroform/*i*-propyl alcohol/acetic acid (94/5/1) and fractions of 45 mL volume are collected. Fractions 15-20 contain pure ketone of formula II (0.082 g, total 0.684 g). Fractions 21-23 contain impure 20 ketone of formula II (0.030 g). Fractions 191-196 contain pure alcohol of formula I (0.151 g).

Fractions 41-63 are pooled and contain primarily alcohol of formula I as a yellow solid (1.34 g). The solid is dissolved in methanol-ethyl acetate and while hot is decolorized with activated charcoal. After filtration and removal of solvent, an off-white solid (1.26 g) remains and is chromatographed over 200 g of 40-63  $\mu$ m silica gel. Fractions of 50 mL volume are 25 collected and fractions 85-106 contain pure crystalline alcohol of formula I (1.0 g).

Recrystallization from acetone-hexane gives alcohol of formula I (0.884 g).

Physical characteristics are as follows:

Mp 154.5-155.5°C.

$[\alpha]_D^{25} -8.1^\circ$  (c, 0.620, ethanol).

30 Fractions 64-77 are pooled and contain a mixture of alcohol of formula I and pioglitazone-N-oxide of formula IV (total, 0.11 g).

Fractions 78-120 are pooled and contain primarily pioglitazone-N-oxide of formula IV (0.282 g). The material is plated onto silica gel (7 g) and placed in line in front of a column of 200 g of 40-63  $\mu$ m silica gel. The system is eluted with chloroform/*i*-propyl alcohol/acetic acid 35 (94/6/1) (2 L) followed by  $\text{CHCl}_3$ /*i*-propyl alcohol/acetic acid (88/10/2). Pure pioglitazone-N-oxide of formula IV (0.186 g) is eluted in fractions 33-42 (45 mL volume).

-10-

Combined extracts 8-10 are dried (sodium sulfate), filtered, and concentrated. The residue is plated onto silica gel (25 g, 40-63 µm), which is placed ahead of a column of 200 g of 40-63 µm silica gel and eluted with *i*-propyl alcohol/ethyl acetate/hexane/acetic acid (5/65/29.5/0.5). Fractions (45 mL volume) 29-40 contain impure alcohol of formula I (0.295 g), 5 which is rechromatographed over 200 g of 40-63 µm silica gel using 5 L of chloroform/*i*-propyl alcohol/acetic acid (94/5/1) followed by a 88/10/2 mixture of the same. Fractions (45 mL volume) 156-176 contain pure alcohol of formula I [0.201 g, total (using the 1.0 g weight from fractions 64-77, above)] 1.35 g.

Physical characteristics are as follows:

10 [α]<sub>D</sub> -6.7° (c, 0.780, ethanol).

The <sup>1</sup>H NMR spectrum of the alcohol of formula I is identical with that of an authentic sample of formula I.

EXAMPLE 2: Characterization of Isolate 02179 (UC 11099) (NRRL 18975)

Materials and Methods:

15 Microbial Strain: UC 11099 was isolated from a soil sample collected in Tennessee. The culture was assigned the Isolate number 02179 in November of 1990, and later it was deposited in the Upjohn Culture (UC) Collection as UC 11099. It was also deposited on 6 May 1992 in the permanent collection of the Northern Research Center, U.S. Dept. of Agriculture, Peoria, IL, under the accession number NRRL 18975.

20 Morphological Characterization: The aerial mycelia of the culture grown on ISP media 2, 3, 4, and 5 (Difco) for 14 days at 28°C were examined directly by light microscopy. Spore surfaces were examined by scanning electron microscopy according to the methods described in A. Dietz, and J. Mathews. 1969. Scanning electron microscopy of selected members of the *Streptomyces hygroscopicus* group. *Appl. Microbiol.* 18: 694-696.

25 Cultural and Physiological Characterization: Methods described in the following references were employed: E. B. Shirling and D. Gottlieb. 1966. Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* 16: 313-340; and A. Dietz and D. W. Thayer (ed.). 1980. *Actinomycete Taxonomy* (Procedures for studying aerobic actinomycetes with emphasis on the streptomycetes). SIM special publication No. 6. Soc. for Indus. Microbiol.

30 Arlington, VA. Observations were made after 14 days incubation at 28°C. Growth temperature range was determined on ISP medium 2.

Cell-wall and Sugar Analysis: Methods described in J.L. Staneck and G.D. Roberts, 1974, "Simplified Approach to Identification of Aerobic Actinomycetes by Thin Layer Chromatography," *Applied Microbiology*, 28:226-231, or methods analogous thereto, were 35 followed to prepare whole cell hydrolysates and to analyze these hydrolysates for amino acid composition and sugar analysis by thin-layer chromatography.

**Results:**

Morphological Characterization: NRRL 18975 produced long spore chains of tight spirals. Spore surfaces were rugose. Vegetative hyphae showed no evidence of fragmentation.

Cultural and Physiological Characterization: The cultural and physiological characteristics of NRRL 18975 on various media are shown in Tables 1 and 2 below. The isolate grew well and produced white aerial mass which became hygroscopic and turned dark gray to black on most media as the incubation time prolonged beyond two weeks. The reverse side of the colony was yellowish brown to yellowish gray. Melanoid pigment was not produced. The culture strongly solubilized tyrosine and weakly solubilized casein. It did not solubilize calcium malate or xanthine. It hydrolyzed starch on ISP medium 4. Nitrate was not reduced to nitrite.

Utilization of carbon sources by NRRL 18975 is shown in Table 3 below.

The isolate grew well and produced white aerial mass which turned dark gray on the positive control (ISP medium 9 plus glucose). A scant black growth was observed on the negative control (ISP medium 9 plus no sugar). The isolate utilized a wide range of sugars, including glucose, arabinose, xylose, mannitol, fructose, rhamnose and raffinose. It did not utilize sucrose or cellulose. Utilization of inositol was questionable.

The isolate grew in the temperature range of 24-45°C with the optimum range of 28-37°C. It did not grow at 55°C.

Cell-wall and Sugar Analysis: The cell-wall of NRRL 18975 contained LL-diaminopimelic acid; no *meso* isomer was detected. The whole-cell hydrolysate contained galactose.

**Conclusion**

Isolate 02179 (UC 11099) (NRRL 18975) has been studied and found to have the macroscopic, micro-scopic and whole-cell hydrolysate properties of the genus *Streptomyces*. The hygroscopic colony appearance and the rugose surfaced spores further place the culture in the species *hygroscopicus*. Therefore, *Streptomyces hygroscopicus* strain 02179 has been determined for NRRL 18975.

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Table 1. Cultural and physiological characteristics of NRRL 18975 on ISP media.<sup>1</sup>

Medium	Parameter <sup>2</sup>	Chip <sup>3</sup>	Color <sup>3</sup>	Comments
<u>Agar plate</u>				
5 ISP medium 2 (Yeast extract - malt extract agar)	G AM R SP	263-266 77 --	white→dark gray medium yellow brown	good
10 ISP medium 3 (Oatmeal agar)	G AM R SP	263-266 93 --	white→dark gray yellow gray	good
15 ISP medium 4 (Inorganic salts-starch agar)	G AM R SP	263-266 77 --	white→dark gray medium yellow brown	good starch hydrolyzed
20 ISP medium 5 (Glycerol-asparagine agar)	G AM R SP	92 93 --	yellow white yellow gray	good
<u>Agar slant</u>				
25 ISP medium 6 (Peptone - yeast extract iron agar)	G AM R SP	93 76 --	yellow gray light yellow brown	good
ISP medium 7 (Tyrosine agar)	G AM R SP	93 92 --	yellow gray yellow white	fair
<u>Broth</u>				
30 ISP medium 8 (Bacto-nitrate broth)				nitrate not reduced to nitrite

<sup>1</sup> All readings taken after 14 days at 28°C.<sup>2</sup> G = Growth                    AM = Aerial mass                    R = Reverse

SP = Soluble pigment

<sup>3</sup> Color was compared with chip standards from the ISCC-NBS (Inter-society Color Council-National Bureau of Standards) color name chart.

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Table 2. Cultural and physiological characteristics of NRRL 18975 on confirmatory media.<sup>1</sup>

	Agar medium	Parameter <sup>2</sup>	Color	Comments
5	Calcium malate	G	No aerial	fair
		AM	light tan	calcium malate not solubilized
		R		none
		SP		
10	Skim milk	G		good
		AM	white	casein weekly solubilized
		R	yellow tan	
		SP		trace of yellow tan
15	Tyrosine	G		good
		AM	white	tyrosine solubilized
		R	yellow tan	
		SP		trace of yellow tan
20	Xanthine	G		good
		AM	white	xanthine not solubilized
		R	tan	
		SP		none
25	Peptone iron	G		good
		AM	white	
		R	tan	
		SP		none
30	Nutrient starch	G		good
		AM	white → dark gray	
		R	yellow tan	
		SP		none

<sup>1</sup> All readings taken after 14 days at 28°C.

35 <sup>2</sup>G = Growth AM = Aerial mass  
SP = Soluble pigment

Table 3. Utilization of carbon sources by NRRL 18975.

	Carbon source <sup>1</sup>	Utilization <sup>2</sup>	Comments
5	D-glucose (positive control)	++	good growth, aerial mass white turning to dark gray
	L-arabinose	++	good growth, aerial mass white turning to dark gray
10	Sucrose	—	no growth
	D-Xylose	++	good growth, aerial mass white turning black
15	i-Inositol	±	scant black aerial mass
	D-Mannitol	++	good growth, aerial mass white turning black
	D-Fructose	++	good growth, aerial mass white turning black
20	Rhamnose	+	fair growth, some black aerial mass
	Raffinose	+	fair growth, some black aerial mass
25	Cellulose	—	very scant growth
	No carbon (negative control)	—	very scant growth

30      <sup>1</sup> Carbon sources were added to ISP medium 9 to give a final concentration of 1%.

<sup>2</sup> ++ = Growth on tested carbon is similar to or greater than growth on positive control (PC).

+ = Growth is somewhat less than PC.

± = Growth is somewhat more than negative control (NC) but much less than PC.

— = Growth is similar to that on NC, or no growth.

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Table 4. Strains of *Streptomyces hygroscopicus*.

	UC No.	Deposit No.
5	5176	ATCC 15869
	5830	ATCC 21840
	5931	NRRL 5491
	8038	IFO 13589
10	8074	NRRL 3999
	5607	ATCC 21582
	5665	ATCC 21589
	5727	NRRL 3576
15	8269	ATCC 31257
	8657	ATCC 31955
	8675	ATCC 39150
	8723	NRRL 15518
20	5208	NRRL 3602
	5549	ATCC 21449
	5623	ATCC 21431
	8029	NRRL 5739
	8625	NRRL 21067

ATCC = American Type Culture Collection; Rockville, MD.

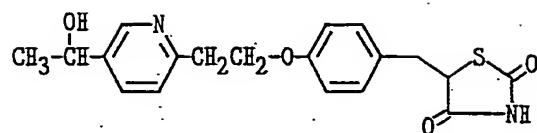
IFO = Institute for Fermentation in Osaka; Osaka, Japan

NRRL = Northern Region Research Center, ARS; Dept. of Agriculture; Peoria, Illinois.

-16-

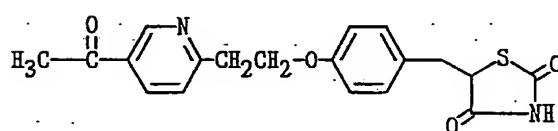
## FORMULA CHART

5



I

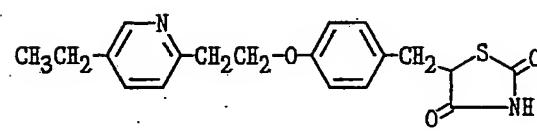
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II

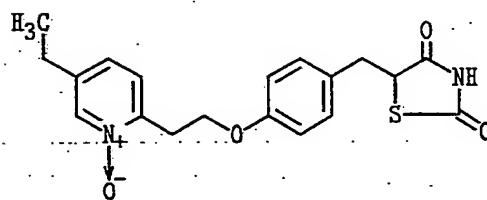
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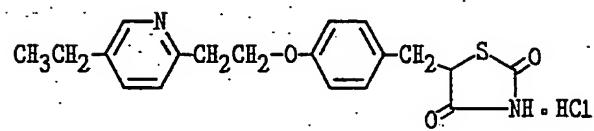
III

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IV

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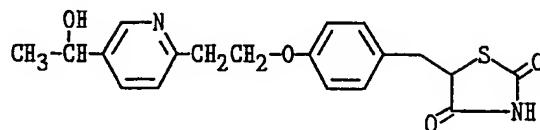


V

## CLAIMS

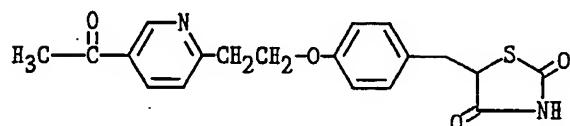
1. A process for producing a compound of the formula I or II:

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I

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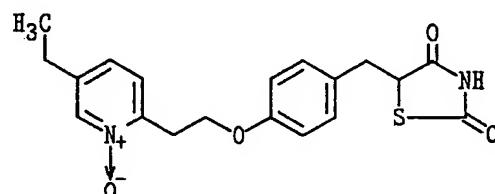
II

which comprises:

the biotransformation of a compound of the formula IV:

15

20



IV

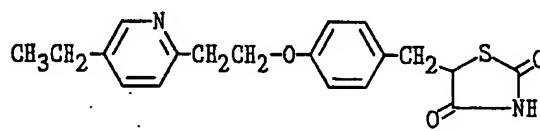
using a microorganism in an aqueous nutrient medium containing an assimilable source of carbon and an assimilable source of nitrogen under aerobic conditions.

25 2. The process of Claim 1 wherein the microorganism is a strain of *Streptomyces hygroscopicus* or a mutant thereof.

3. The process of Claim 2 wherein the strain is NRRL 18975 or a mutant thereof.

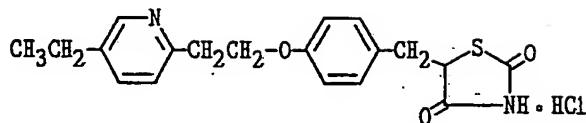
30 4. The process of Claim 3 which further comprises the biotransformation of a compound of the formula III:

35



III

or of a compound of the formula V:



5

to produce the compound of formula I or II.

5. The process of Claim 4 which further comprises isolating the compounds of formulas I and II which are 5-[4-[2-[5-(1-hydroxyethyl)-2-pyridyl]ethoxy]benzyl]-2,4-thiazolidinedione and  
10 5-[4-[2-(5-acetyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione.

15 6. The process of Claim 1 wherein the biotransformation is conducted at a temperature of from about 23°C to about 32°C.

15 7. The process of Claim 6 wherein the temperature is about 28°C.

15 8. The process of Claim 1 wherein the biotransformation is conducted at a pH of from about 6.5 to about 7.5.

20 9. The process of Claim 8 wherein the pH is about 7.2.

20 10. The process of Claim 1 wherein the nutrient medium contains carbon sources of from about 1 to about 5% by weight and nitrogen sources of from about 1 to about 5% by weight.

25 11. The process of Claim 4 wherein the biotransformation is complete in about 1 to 4 days after the addition of the compound of formula III or the compound of formula IV to a growing culture of the microorganism.

30 12. The process of Claim 11 wherein the biotransformation is complete in about 1.5 days.

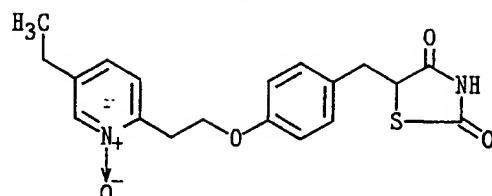
13. A biologically pure culture of the microorganism *Streptomyces hygroscopicus*, characterized as the strain NRRL 18975.

35 14. A method of using a compound of the formula IV:

35

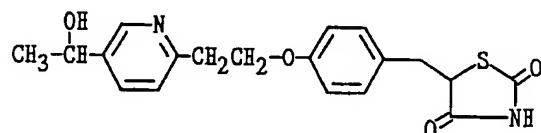
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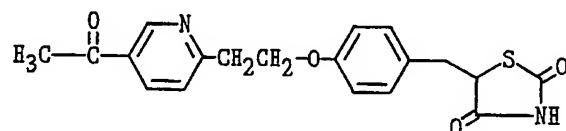


to produce a compound of the formula I or II:

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which comprises:

adding the compound of formula IV to a metabolizing culture of a microorganism.

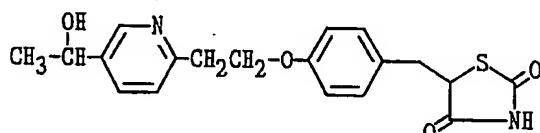
15. The method of Claim 14 wherein the microorganism is *Streptomyces hygroscopicus*,  
20 strain NRRL 18975, or a mutant thereof.

16. The method of Claim 15 which further comprises isolating the compounds of formulas I  
and II which are 5-[4-[2-[5-(1-hydroxyethyl)-2-pyridyl]ethoxy]benzyl]-2,4-thiazolidinedione and  
5-[4-[2-(5-acetyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione.

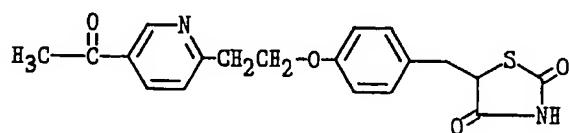
25

17. A method of using a microorganism *Streptomyces hygroscopicus*, strain NRRL 18975,  
or a mutant thereof, to produce a compound of the formula I or II:

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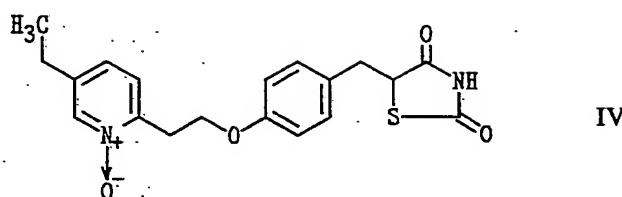


which comprises:

-20-

adding a compound of the formula IV:

5

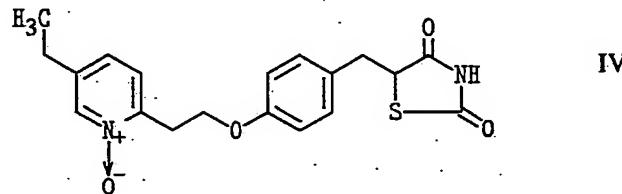


to a metabolizing culture of the microorganism.

10 18. The method of Claim 17 which further comprises isolating the compounds of formulas I and II which are 5-[4-[2-[5-(1-hydroxyethyl)-2-pyridyl]ethoxy]benzyl]-2,4-thiazolidinedione and 5-[4-[2-(5-acetyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione.

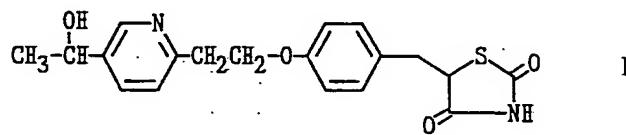
15 19. A method of deoxygenating the N-oxide of a pyridine ring and oxygenating the 5-position ethyl side chain of the pyridine ring in a compound of the formula IV:

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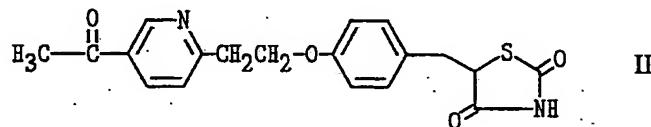


to produce a compound of the formula I or II:

25



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which comprises:

adding the compound of formula IV to a metabolizing culture of a microorganism.

35 20. The method of Claim 19 wherein the microorganism is *Streptomyces hygroscopicus*, strain NRRL 18975, or a mutant thereof.

-21-

21. The method of Claim 20 which further comprises isolating the compounds of formula I and II which are 5-[4-[2-[5-(1-hydroxyethyl)-2-pyridyl]ethoxy]benzyl]-2,4-thiazolidinedione and 5-[4-[2-(5-acetyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione.

## INTERNATIONAL SEARCH REPORT

PCT/US 93/03569

International Application No.

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.C1. 5 C12P17/16; C12N1/20; //((C12N1/20,C12R1:55))		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.C1. 5	C12P ; C12R ; C07D	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	EP,A,0 257 781 (TAKEDA) 2 March 1988 see claims	1
A	ARZNEIMITTEL FORSCHUNG - RESEARCH vol. 40, no. 1, 1990, AACHEN/DORF DE pages 37 - 42 T. SOHDA ET AL. 'Studies on antidiabetic agents. Synthesis and hypoglycemic activity of 5-[4-(pyridylalkoxy)benzyl]-1,4-thiazolidinediones.' see the whole document	1
A	FR,A,1 365 278 (ROUSSEL-UCLAF) 25 May 1964 see the whole document	1
	---	-/-
<p><sup>10</sup> Special categories of cited documents :<sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <b>05 AUGUST 1993</b>	Date of Mailing of this International Search Report <b>24-08-1993</b>	
International Searching Authority <b>EUROPEAN PATENT OFFICE</b>	Signature of Authorized Officer <b>DELANGHE L.L.M.</b>	

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	<p>DATABASE WPI        Section Ch, Week 8737,        Derwent Publications Ltd., London, GB;        Class B02, AN 87-260936        &amp; JP,A,62 181 275 (PHARMACEUTICAL) 8        August 1987        see abstract</p> <p>---</p>	1
P,A	<p>EP,A,0 508 740 (TAKEDA)        14 October 1992        cited in the application        see claims</p> <p>---</p>	1
P,A	<p>WO,A,9 218 501 (THE UPJOHN COMPANY)        29 October 1992        cited in the application        see claims</p> <p>-----</p>	1

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9303569  
SA 73718

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 05/08/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0257781	02-03-88	DE-A-	3773089	24-10-91
		JP-A-	63139182	10-06-88
		US-A-	4812570	14-03-89
		US-A-	4898947	06-02-90
FR-A-1365278		None		
EP-A-0508740	14-10-92	JP-A-	5092972	16-04-93
		US-A-	5183823	02-02-93
WO-A-9218501	29-10-92	AU-A-	1743292	17-11-92
		JP-A-	5086057	06-04-93